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RESEARCH ARTICLE

CO-ADMINISTRATION OF ARTESUNATE AND AZADIRACHTA INDICA EXTRACT: EFFECTS ON ANTIOXIDANTS AND CERTAIN LIVER PARAMETERS IN MALE WISTAR RATS

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ABSTRACT

Resistance to artemisinins has led to possible anti malarial combination therapy. This study was undertaken to investigate the effects of co-administration of artesunate and *Azadirachta indica* leaves extract on antioxidants and certain liver parameters in rats. 24 male rats weighing averagely 190g were randomly selected into four groups, administered therapeutic doses of artesunate and extract namely : Group A (Control), Group B (Extract only)), Group C (Artesunate only) and Group D (Artesunate with extract). Total protein, Cholesterol concentrations, Alanine amino transferase (ALT), Alkaline phosphatase (ALP) and Gamma glutamyl transferase (GGT) activities were determined using international standardized methods. Malondialdehyde, (MDA), reduced glutathione (GSH), phenols, flavonoids, hydroxyl (OH^-) and 1-1-diphenyl-2-picryl hydrazyl (DPPH) radicals scavenging effects were determined spectrophotometrically. Results obtained showed significant increases ($P \leq 0.05$) in plasma total protein concentrations in all treatment groups compare with controls while a modulatory effect was observed with plasma total cholesterol concentrations as the combined treatment (Group D) reduced this to controls. Artesunate alone elicit significant increases ($P \leq 0.05$) in plasma ALT,ALP and GGT activities while the combined treatment group D showed a modulatory effect as these activities were significantly decreased ($P \leq 0.05$) nearly to control level. Interestingly rats challenged with artesunate shows significant decreases ($P \leq 0.05$) in GSH level compared with group A,B and D while MDA level were significantly decrease ($P \leq 0.05$) by group B, C and D compared with controls . Extract shows considerable amount of phenol and flavonoids and also scavenged (OH^-) and DPPH radicals by 72.81% and78.60% at 350µg/ml concentrations in-vitro. Results are suggestive of hepatoprotective and modulatory effects of extract on artesunate which may affects its mechanism of action or synergistically enhance its anti malarial activity, an indication that the plant contain bioactive agents which may be characterized for development of drugs in combating *Plasmodium falciparum* resistance.

INTRODUCTION

Malaria is one of the major infectious diseases responsible for the high rate of mortality and morbidity in developing countries caused by an apicomplexan parasites belonging to the genus Plasmodium. Four species of plasmodium parasites are known to cause infection in humans; Plasmodium falciparum, *P. Vivax, P. Ovale* and *P. Malariae*. Plasmodium Falciparum is the most virulent parasite and is highly incriminated in the global mortality rate of malaria infection .It is the most common human parasite in Africa, and is seen in all malaria endemic regions of the world (Miller *et al*., 1994; Singh *et al*., 2004; WHO, 2005). The problem of malaria resistance to its major classical drugs continue to pose challenges to treatment of malaria especially in some poor countries of Africa. Classical drugs such as chloroquine, sulphadoxine pyrimethamine as well as artemisinins are currently been resisted by the malarial parasites especially the Plasmodium Falciparum specy of the parasite hence the need for current and rigorous search for novel anti malarial drugs to overcome resistance (Ridley, 2002). The wide spread and increasing occurrence of *P. falciparum* resistant against affordable antimalaria drugs like chloroquine (CQ) and sulphadoxine primenthamine (SP) is more and more

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hampering the fight of these drugs against malaria. CQ and SP are still the most widely used drugs for malaria treatment in most African countries due to their low cost and availability. Also the usage of commbination therapy preferably artemisinin – based combination of antimalaria drugs, one of which is an artemisin derivature e.g artesunate, artemether or dihydroartemisin and amodiaquine is currently approved by WHO to overcome resistance (Looarwsuwan *et al*., 1992). Artemisinin containing combination therapies will undoubtedly increase in their usage and perhaps in their public health importance with time as resistance becomes more widespread to a wider range of single anti-malarial drugs especially after treatment with artemisinins (Ridley, 2002).

The anti malaria drugs quinine and artemisinins are both plant derivatives suggesting that other effective malaria drugs might be plant –derived while traditional remedies may still be the best and alternative means of overcoming resistance in the tropics. In this way it is pertinent to note that a number of drugs currently in clinical use exert their activities at least in part by increasing oxidative stress in the parasitized erythrocytes while ROS are often produced by the host immune system adds to the overall oxidative burden of the parasitized cell (Becker *et al*., 2004). However based on this evidence of oxidative stress associated with malaria chemotherapy, this study attempt to explore the possibilities of using known anti malarial plants which have been found to contain certain bioactive agents with antioxidants activities as a possible modulatory agent on the activities of these drugs as a possible means of an attempt to overcome drug resistance in malaria chemotherapy using combinative therapy especially with artemisinins based drugs as the justification for this study. Azadirachta indica plants commonly known as Neem and its extracts from the Meliaceae family is a common household medicinal plant in the tropics where it is used extensively as a traditional remedy against malaria while other laboratory works have shown the efficacy of the plants extracts as potent inhibitor of the Plasmodium Falciparum asexual stages and are effective in its anti–plasmodial activity especially with the *P.Falciparum* (Leaman *et al*., 1995; Lucantoni *et al*., 2010). This study therefore examined the possible effects of co-administration of methanol extract of azarachta indica and artesunate on certain antioxidant and liver indices in rats with the aim of assessing the link and possible effect of the extract on the drug action.

MATERIALS AND METHODS

Materials

Some of the materials used for this study includes: measuring cylinder, electronic balance, breakers, test – tubes, testtube racks, waterbath, dissecting set, disposable gloves, centrifuge, thermometer, micropipettes, spectrophotometer, tissue paper, spatula, needles, syringes, thumb pin, washing brush, film container, detergent, stop watch, refrigerator, blade, homogenizer, cotton wool, cuvette, sample bottles, blender, plant materials, artesunate tablet e.t.c.

Reagents

Methanol, normal saline, laboratory kits for analysis of total protein, total cholesterol, AST, ALT, GGT, sodium hydroxide, sodium chloride, potassium chloride, thiobarbituric acid, DPPH, Folin Secateau reagent, Tris buffer and Trichloroacetic acid all of which are of good analytical grade and were obtained from Sigma Chemicals USA.

Plant Materials and Preparation of methanol extract.

The leaves of Azadirachta indica (family: Meliacceae) were obtained within the premises of the college of Health Sciences of my institution, identified and authenticated at the Botany Unit of the Department of Pure and Applied Biology with the herbarium voucher number LHO214 deposited. Methanol plant extracts were obtained from fresh healthy leaves of the plant which were air –dried, powdered, weighed and soaked in cold methanol for 72 hours in the dark after which the filtrate was concentrated at 40^0C to obtained the dry methanol extract.

Phytochemical study

Determination of the total phenolic contents of the extracts was based on the reduction of Folin-ciocalteau reagent (Phosphomolybdate and Phosphotungstate) by the method of Mc Donald *et al*., 2001. The reduced folin-ciocalteau reagent is blue and the absorbance was read at 750nm, while results were obtained in garlic acid equivalence using the various concentrations of garlic acid as the standard. The total flavonoids content were estimated using Aluminium Chloride colourimetric method by Chang *et al*., 2002 based on the principle that flavonoids form complexes with Aluminium Chloride. This was determined as quercetin equivalence from a standard curve obtained at different concentrations of quercetin.

Antioxidants Activity

Antioxidants activities studied includes, the free radical scavenging activities determined using the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) by the photometric method of Mensor *et al*., (2001), with ascorbic acid as standard and the hydroxyl radicals scavenging activity performed as described by Halliwell, (1987), using iron/EDTA/H_2O_2 complex. Malondialdehyde levels and reduced glutathione (GSH) determinations were carried out by the methods of Varshney and Kale (1990) and Beutler *et al*., (1963).

Experimental Animals

Twenty four healthy male Wistar rats were obtained from the animal house of the University of Ibadan. They were randomly allocated into four groups A, B, C, and D with six rats per group. They were acclimatized with the same environmental condition fed ad-libitum in a well ventilated plastic cage for six weeks in the animal house of the Department and handled based on the regulations and guard lines for handling laboratory animals by my institution.

Experimental design and administration of test substances

Artesunate was administered at 2mg/kg body weight orally as its standard dosage and this was applied to the administration of the extract based on the average weight of the experimental animals (190g). Hence four treatment groups (A, B, C, and D). were identified based on the experimental design and treated

as follows; The control (Group A) given 0.1ml normal saline intravenously two times in a day (morning and evening)for three consecutive days). Group B; administered orally with methanol extract of Azadirachta indica (in 0.1ml corn oil containing 0.38mg of extract) twice a day for three days while, 0.1ml containing 0.38mg of drug (Artesunate) was administered orally to the rats in Group C twice a day for three days. Group D (combinative therapy or treatment) was also administered with 0.1ml of each drug and extract separately and simultaneous through oral means of route of administration, twice in a day for three consecutive days.

Collection of Blood Sample and preparation of liver homogenate

Rats from all the groups were sacrificed by cervical dislocation. Blood was collected directly from the heart into plain and well-labelled sample bottles containing heparin anticoagulant and were centrifuged at 4000rpm for 5 minutes to obtain plasma for analysis of biochemical parameters. The hepatic tissues were homogenized in KCl [10 mM] phosphate buffer (1.15%) with ethylene-diamine tetra acetic acid (EDTA, pH 7.4) and centrifuged at 12, 000 rpm for 60 min. The supernatant was used to assay for reduced glutathione and malondialdehyde concentrations.

Biochemical indices

Protein and cholesterol concentrations were determined by the procedure of Lowry *et al*., (1951) and Trinder, (1969). Enzyme assays, Alanine Aminotransferase (ALT), Alkaline Phosphatase (ALP) and Gamma glutamyl transferase (γGT) were determined using the method of Wroblwski et al., (1956); Burtis *et al*., (1999) and Szasz, (1969) based on the standardized methods by the International Federation of Clinical Chemistry.

Statistical Analysis

Statistical analysis were based on the Duncan`s experimental analysis with mean standard deviation of sample analysed using the student T test and $p< 0.05$ i.e 95% level of significance or confidence limit (Bliss, 1967).

RESULTS AND DISCUSSION

The alarming rate at which Plasmodium Falciparum has developed resistance to chloroquine, artesunate and other synthetic anti malarial drugs has made it necessary for search for effective anti malarial compounds and in areas where malaria is endemic, traditional medicinal plants are mostly used to cure or treat malaria hence further investigation of green plants must be continuous to overcome resistance in malaria chemotherapy. Artesunate has been found to be ideal for treatment of severe malaria including cerebral malaria and as a potent blood scizonticide agent for P. Falciparum. However with its rapid anti parasitic activity, it has short half lives such that the standard 3-day treatment course is commonly followed by recrudescence of infecting parasites and recurrent illness within days to weeks hence this form of resistance are presently faced with addition of long –acting agent known as artemisinin-based combination therapy as a means of overcoming this resistance (Woodrow *et al*., 2005) Also, artesunate is a derivative of artemisinin which is obtained from the plant Artemisia annua while its possible toxicity such as neurotoxicity and reproductive toxicity has been reported (Woedenbag *et al*., 1994; Li *et al*., 2006 and Clark *et al*., 2008). From this study the effects of co-administration of artesunate and methanol extract of *Azadirachta indica* on antioxidant and certain liver parameters in rats was investigated to have a clue about the possibility of the extract ameliorating the drug toxicity and or modulate the anti malarial activity of the drug through combinative therapy to overcome resistance since herbal medicines have been reported to be safe and without any adverse side effect when compared with synthetic drugs and that certain bioactive substances present in them have been shown to produce certain physiological action on the human body. From the results obtained all the treated groups significantly increase ($P\leq0.05$) plasma total protein concentrations compared with the controls (table 1). The increases might results from the ability of artesunate and *Azadirachta indica* to enhance the synthesis of Protein as earlier reported by Udayashekara, (1987). However in table 1, animals treated with artesunate alone (group C) significantly increase ($P\leq0.05$) total plasma cholesterol concentrations compared to the control, suggestive of artesunate toxicity as reported earlier (Meshnick, 2002; Clark *et al*., 2008), while animals treated with both extract and artesunate (group D) shows a modulatory effect on the artesunate activity by the extract as the cholesterol was restored below the control level.

Behaviour exhibited by the extract further supported other reports on the possible antihypertensive, anti-hyperlipidemic activity and anti-inflammatory properties of the extract (Pendse *et al*., 1977; Chattopadhyay *et al*., 1993; Chattopadhyay, 1998; Muregi *et al*., 2003). The effects of artesunate on certain liver enzymes shows that the drug significantly increase ($P\leq0.05$) the activities of plasma alkaline phosphatase (ALP), alanine amino transferase (ALT) and gamma glutamyl transferase (GGT) compared with the controls (table. 2), however the extract modulate the activities of these enzymes as they were reduced close to their control levels by the combined treatment (group D). The elevated levels of these enzymes may not be unconnected with the possible injury to the liver by the drug (Meshnick, 2002; Li *et al*., 2006). Interestingly, the antioxidant indices investigated in this study showed that the extract elicit significant increase ($P\leq0.05$) in the liver GSH concentrations compared to control and other treatment groups (Table.3). The artesunate treated animals however showed a significant decrease ($P\leq0.05$) in GSH concentration compared with other treatments group as the combined treatment increase the GSH level significantly ($P\leq0.05$) compared with artesunate group. The antioxidant property shown by the extract in increasing tissue GSH singly and both in combination with artesunate are attributes of its possible effects on the anti-malarial activities of the drug and its pharmacokinetic profile. Similarly both extract and artesunate administered singly or combined significantly decrease MDA concentrations in the liver below the control level (group B,C and D). The ability of the extract to reduce MDA level is an indication of the antioxidant potential of the extract to maintain membrane integrity. Further in-vitro antioxidant analysis of the extract showed considerable amount of phenol and flavonoids which were expressed in a concentration – dependent manner (Table 4), ranging between

Table 1. Plasma protein and cholesterol concentrations of various treatment groups.

Treatment group	Protein concentrations (mg/dl)	cholesterol concentrations (mg/dl)
A (Control)	520.14±34.70	264.12±9.39
B (Extract only)	568.70±90.60	273.57±53.80
C (Artesunate only)	893.52±242.65	307.00±36.52
D (Artesunate + extract)	661.81±37.07	235.99±50.99

*Values are given as mean and standard deviation of six determinations with P≤0.05 as level of significance.

Table 2. Plasma Alanine amino transferase (ALT), Alkaline phosphatase (ALP) and Gamma glutamyl Transferase (GGT) activities in various treatment groups.

Treatment group	ALT Activity (IU/L)	ALP Activity (IU/L)	GGT Activity (IU/L)
A(Control)	0.21±0.11	7.26±1.33	2.70±0.07
B (Extract only)	0.22±0.11	8.09±2.39	3.47±0.01
C (Artesunate only)	0.27±0.06	12.00±1.32	4.24±0.77
D (Artesunate + extract)	0.20±0.01	10.47±1.10	3.86±1.51

* Values are given as mean and standard deviation of six determinations with P≤0.05 as level of significance.

Table 3. Reduced Glutathione (GSH) and Malondialdehyde (MDA) concentrations in various treatment groups

Treatment group	Reduced Glutathione GSH(Mg/ml protein)	Malondialdehyde MDA(Mg/ml)
A(Control)	995.15 ± 66.16	3.05 ± 0.35
B (Extract only)	1125.70 ± 28.7	2.5 ± 0.12
C (Artesunate only)	787.80 ± 59.47	2.31 ± 0.26
D (Artesunate + extract)	861.20 ± 47.29	2.61 ± 0.29

*Values are given as mean and standard deviation of six determinations with P≤0.05 as level of significance.

Table 4. Total phenolic and flavonoids contents of the methanol extract of Azadirachta indica leaves at various concentrations in gallic acid and quercetin equivalence.

Concentrations Mg/ml	Total phenolic content Gallic acid equivalent.GAE (Mg/g)	Total flavonoids content Quercetin equivalent QE (Mg/g)
100.00	0.005 ± 0.001	0.001 ± 0.0001
200.00	0.007 ± 0.014	0.002 ± 0.0005
300.00	0.008 ± 0.001	0.003 ± 0.0004
400.00	0.010 ± 0.003	0.004 ± 0.0008
500.00	0.011 ± 0.003	0.005 ± 0.0001
600.00	0.014 ± 0.027	0.006 ± 0.0002
700.00	0.015 ± 0.002	0.007 ± 0.0004

*Mean Value ± Standard deviation of three replicates.

Table 5. Percentage scavenging activities of DPPH and Hydroxyl radicals by various concentrations of Azadirachta indica leaves extract in-vitro.

Concentrations (µg/ml)	% DPPH SCAVENGING EFFECTS ±SD	% HYDROXYL RADICAL SCAVENGING EFFECTS±SD
50.00	6.24 ± 0.019	44.00 ± 0.004
100.00	21.34 ± 0.043	50.00 ± 0.002
150.00	40.10 ± 0.004	62.00 ± 0.001
200.00	46.28 ± 0.080	64.00 ± 0.002
250.00	72.14 ± 0.006	68.00 ± 0.003
300.00	74.49 ± 0.035	72.00 ± 0.004
350.00	78.60 ± 0.057	72.79 ± 0.004

*Mean Value ± Standard deviation of three replicates.

0.005-0.015 mg/g Gallic acid equivalence and 0.001-0.007 mg/g Quercetin equivalence respectively. The presence of this compounds accounts for its antioxidant property as most phenolic compounds have been found to be predominantly in plants with antioxidant properties and high impact on health (Dimitrios, 2006). Also of note is the ability of the extract to scavenge free - radicals in-vitro as it scavenged DPPH and Hydroxyl radicals by 78.60 % and 72.79% at 350µg/ml concentrations respectively. The in-vitro antioxidant results correlate with the properties exhibited by the extract in-vivo when used in combination with artesunate suggesting that the extract may have a different pharmacokinetic profile as an antioxidant from artesunate which induces oxidative stress to eliminate the parasite.

However since most antioxidants interact with and stabilize free radicals and may prevent some of the damage free radicals otherwise might cause as well as bolster the body's own defenses against biological invaders transmitted by germs warfare, mosquitoes or other exposures. This property may be explore as a clue to a possible mechanism to overcome resistance when the bioactive agents of this extract are used in combination with artesunate.

Conclusion

Conclusively, results from this study shows modulation on artesunate toxicity and possibly its anti malarial mode of action by the administered extract which may account for the

bioactive constituents of the plants which can be explore for further research on overcoming drug resistance to P. falciparum malaria. Also, further research should be carried out to identify or isolate the active compounds or compounds responsible for these effects which could be helpful in drug design and development.

Disclosure of conflict of interest

There are no conflicts of interest between authors.

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